THE INFLUENCE OF LIGHT ON CELL AGGREGATION IN POLYSPHONDYLIUM PALLIDUM

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The effect of light on aggregation in the cellular slime molds has been known since Potts (1902) observed that *Dictyostelium mucoroides* formed more numerous and smaller aggregates when incubated in the light than in the dark. Harper (1932) and Raper (1940) made similar observations on *Polysphondylium violaceum* and *D. discoideum*, respectively, while Konijn and Raper (unpublished data) found comparable phenomena to exist in *P. pallidum* and *D. purpureum*. The influence of light on cell aggregation is not limited to the more complex species; for example, Olive and Stoianovitch (1960) found that in the absence of light the myxamoebae of *Acrasis rosea* fail to develop beyond the vegetative stage and generally undergo encystment. This observation has been extended by Reinhardt (personal communication) to show that it is the relationship between light *and* dark, rather than exposure to light, that is important in the development of this organism.

In the present study, the effect of light on cell aggregation and aggregation density (*i.e.*, the number of aggregates per unit area) in *Polysphondylium pallidum* has been examined in some detail with particular attention to the nature of the light response. The process of aggregation has been observed, *in situ*, under natural conditions of growth, and as it is affected by various modifications in the cultural environment. Based on these observations and experiments, and those of other authors, a hypothesis is presented which may account for the effect of light and may help to explain certain features of aggregation such as the time of onset and the number of centers per unit area.

Materials and Methods

The species investigated was *Polysphondylium pallidum* Olive, strain WS-320. The stock cultures were maintained on dilute hay-infusion agar (Raper, 1951) in association with *Escherichia coli*, strain B/r. For the majority of experiments, the myxamoebae were grown on a suspension of *E. coli* cells in *M*/60 phosphate buffer, following Hohl's and Raper's (1963) modification of Gerisch's technique (1960), and harvested in the stationary phase. The cells were washed essentially free of bacteria by differential centrifugation, and were resuspended in Bonner's salt solution (Bonner, 1947) to a concentration of 2×10^6 to 1×10^7 cells/ml. and dispensed in 0.01-ml. drops on an agar substrate consisting of 1.5% Bacto purified agar in Bonner's salt solution. It is most important that the agar be prepared and

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poured into Petri dishes 40 to 48 hours prior to use, to allow the evaporation of the excess liquid. If this is done, the liquid from the drop will enter the agar within about an hour, leaving a circle of uniformly distributed myxamoebae. Such circles cover an area, on the average, of from 10 to 12 $mm.^2$, thereby yielding cell densities on the substrate of from 1.7×10^3 to 1×10^4 cells per mm^2.

The plates were incubated at 20° C. and scored for the number of centers of aggregation per drop after 22 to 26 hours. Illumination when required was provided by a single 15-watt daylight fluorescent bulb (maximal emission between 430 and 615 $m\mu$) placed at a distance of 22–25 cm. from the cultures and giving an intensity of about 75 foot-candles at the level of the agar. In other experiments conducted under either "dark" conditions or requiring dark-grown myxamoebae, all procedures (centrifugation, dispensation of aliquots, *etc.*) were carried out in a room dimly lit with a Kodak safelight equipped with a red Wratten filter. Darkness during incubation was maintained by keeping the plates in light-tight Petri dish canisters.

For studies of cell growth and aggregation on the same plate, Petri plates containing a nutrient gel (0.3% lactose, 0.15% peptone, and 1.5% agar, phosphate buffered to pH 6.0–6.2) were spread with a suspension of bacteria and inoculated in the center with spores of *P. pallidum*. Incubation was at 20° C.

Results

In the first series of experiments, myxamoebae were grown in the dark in liquid culture, processed (harvested, washed, *etc.*), and exposed to light for various lengths of time following deposition on agar, and then returned to the dark. The results of two such experiments are shown in Figure 1.

The outstanding feature of this graph is the increase shown in the number of centers formed by the myxamoebae with increasing exposure to light. For the two experiments in Figure 1, this amounted to a 6-fold (upper curve) and a 12-fold (lower curve) difference in the number of centers for cultures under continuous illumination, while in still another experiment a 31-fold difference was observed. Not only is there an increase in the number of centers, but the interval between the time of deposition of the drops and the onset of aggregation is shortened by exposure to light. This "hastening" of aggregation by light has been examined in some detail by Raper (1940), Shaffer (1958) and Konijn and Raper (unpublished data). Clearly, then, light can play a major role not only in determining the number of centers that will form in a population of myxamboebae, but also in hastening the time at which they appear. When is light most effective? From these experiments and some that follow, it appears that light becomes effective shortly after the deposition of the drops, generally within the first two to three hours of incubation. During this period, even a comparatively short exposure to light is quite effective in increasing the number of centers.

The degree of sensitivity of the light response mechanism is indicated by the fact that one minute of light appeared to be almost as effective in inducing center formation as were 120 minutes (Fig. 2). In other words, under the conditions of these experiments, a small amount of light at the "right time" was particularly effective in increasing the number of centers. Note also the difference in the level

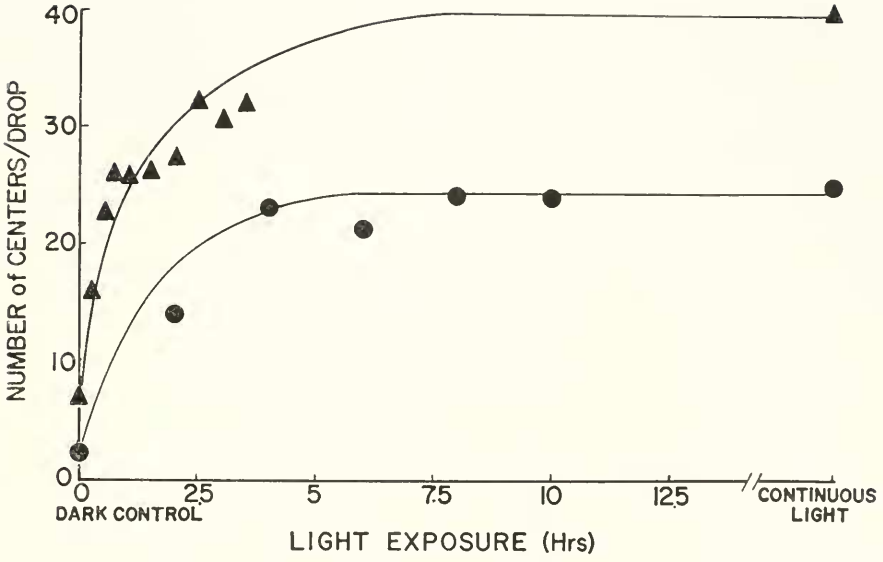

FIGURE 1. The number of aggregation centers formed per drop is plotted as a function of exposure to light. The myxamoebae were grown in the dark, deposited on agar, and exposed to light; they were then returned to the dark following various time intervals. Counts of the number of centers were made after 24 to 26 hours of incubation. Each point represents the mean of 15 to 20 values. The two curves represent different experiments.

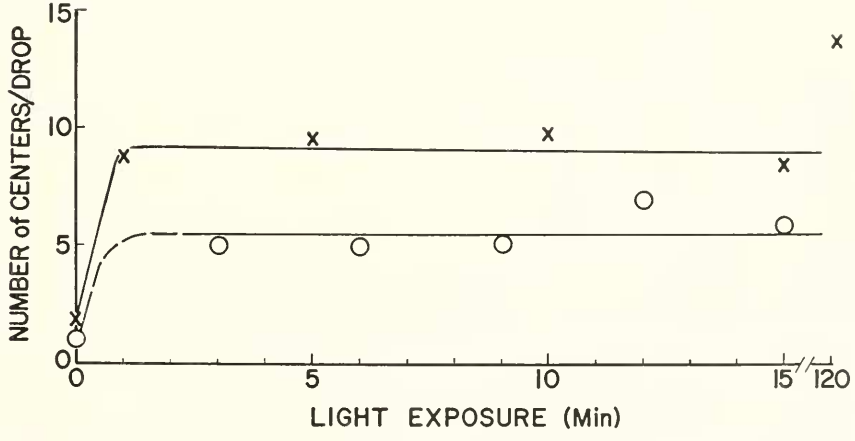

FIGURE 2. The effect of varying light exposure on aggregation. Dark-grown myxamoebae were exposed to light for from 3 to 15 minutes in one case (O) and from 1 to 120 minutes in the other (X); they were then returned to dark. In the latter experiment (X), the light exposure was made after dark incubation of two hours; in the former (O), the exposure followed four hours of dark incubation. Note that the values after four hours are less than those after two hours. Each point represents the mean value of 15 to 20 replicate drops.

of response between the two experiments illustrated in Figure 2. In the upper curve, the myxamoebae were exposed to light after two hours of dark incubation, in the lower, after four hours. This difference in responsiveness would suggest a loss in light sensitivity during prolonged dark incubation. To check this possibility, dark-grown myxamoebae were exposed to 15 minutes of light at various times during incubation and then returned to the dark. The results of three experiments are shown in Figure 3.

Two features are apparent in these experiments. One, populations of myxamoebae which receive light form a greater number of centers of aggregation than those maintained in darkness. Two, the effectiveness of light diminishes during incubation, *i.e.*, cultures that receive light early during incubation form a greater

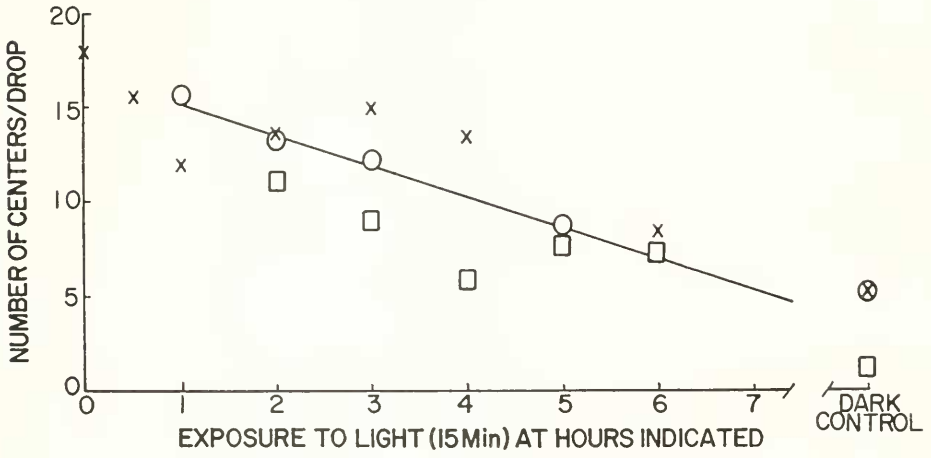

FIGURE 3. The effect of short-term light exposure on aggregation. Dark-grown myxamoebae were exposed to light for single 15-minute periods at various times during incubation and then returned to the dark. Scoring for the number of centers took place 24 hours after the deposition of the drops. Each point represents the mean of 15 to 20 values. The symbols X, O, and □ represent three different experiments.

number of centers than similar cultures that receive the same amount of light later. Indeed, cells which are not incorporated into aggregates during the period of maximum sensitivity may never aggregate, even when exposed to light for much longer periods. These unincorporated cells form microcysts, *i.e.*, encysted individual myxamoebae. (For a discussion of the encystment stages of cellular slime molds see Blaskovics and Raper, 1957.)

Studies of myxamoeba populations grown in situ

Up to this point, we have examined the influence of light on isolated populations of pregrown myxamoebae deposited on non-nutrient agar. It seemed of interest, therefore, to know the response of myxamoebae under more routine culture conditions. For such observations agar plates containing 0.3% lactose-0.1% peptone as nutrient were spread with a suspension of *E. coli* B/r and inoculated in the center

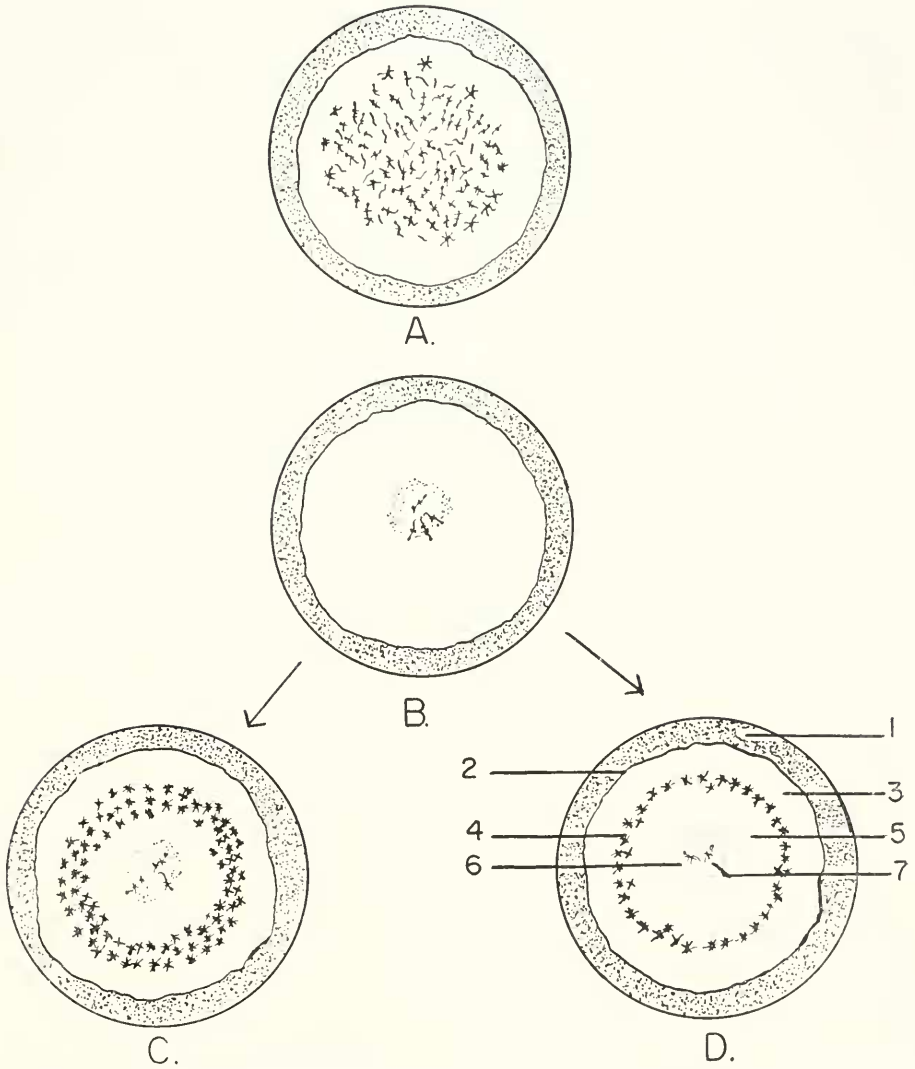

FIGURE 4. Diagram illustrating the behavior of cultures grown *in situ* under conditions of continuous light (A), continuous darkness (B), and after exposure of dark-grown cultures to light (C), and to charcoal (D). Note the differences in the levels of development in the light- and charcoal-treated cultures: (1) unconsumed bacteria, (2) feeding front (bacteriophagic activity), (3) "young" post-feeding myxamoebae, (4) aggregation, (5) "old" post-feeding myxamoebae, (6) microcysts, and (7) limited sorocarps at or near the point of inoculation which may or may not be produced.

with spores of *P. pallidum*. When incubated in the light such plates show a progression of growth and subsequent morphogenesis from the point of inoculation to the periphery of the agar surface (Fig. 4, A). Plates, similarly prepared but inoculated in the dark, sometimes show the presence of sorocarps at or near the point of

inoculation but fail to display any further sign of fructification by the remaining myxamoebae (Fig. 4, B). The fact that some sorocarps do develop is of some interest. For the moment, suffice it to say that this apparently does not result from some center-inducing factor carried by the spores which could initiate aggregation.

If the plates prepared in the manner described above are incubated in the dark for 5 or 6 days and then exposed to light, center formation will occur only within a limited circular zone. The remainder of the plate shows, from the point of inoculation to the margin, areas in which the following developmental stages may be seen: sorocarps (usually less than 10), microcysts, "old" myxamoebae, aggregations, or sorocarps, "young" post-feeding (interphase) myxamoebae, feeding myxamoebae, and bacteria (Fig. 4, D). By marking the daily increment of growth as the colony expands, and correlating "age" with the time of light exposure, it has been determined that the myxamoebae in the zone of aggregation or culmination are two to three days old. Furthermore, a much greater exposure to light (hours instead of minutes) is required to induce aggregation in these cells than in the isolated populations previously discussed. Older cells, under these conditions, show no signs of activity. On the basis of this difference in behavior, it would appear that, as in isolated populations, the ability to aggregate as a response to light is limited in time, *i.e.*, to rather "young" myxamoebae.

The same type of response occurs if similar cultures incubated in continuous darkness are exposed to vegetable charcoal (*cf.* Bonner and Hoffman, 1963). That is, a band or zone of sorocarp formation and cell aggregation appears near the periphery of the expanding colony. Moreover, this zone often appears more quickly, is of greater width (*i.e.*, encompasses still "older" areas of myxamoebae), and shows a greater density of aggregation centers than in comparable cultures exposed to light (Fig. 4, C). But more than this, the myxamoebae within the broader zone respond at about the *same* time. This "synchrony" is also evident in light-induced cultures, but is less striking because the responding myxamoebae are more nearly of the same age. In charcoal-treated cultures, the unaggregated myxamoebae within a zone may differ in chronological age by a matter of *four* to *five* days. It is as if the myxamoebae, upon reaching a certain stage of development, underwent a developmental "freeze" which kept them primed for aggregation for several days. This is in contrast to (1) isolated populations which show, over a short period of time, a decline in the ability to aggregate as a response to light (Fig. 3), and to (2) cultures grown *in situ* in the light which show continuous morphogenetic activity in the zone between the feeding front and the mature sorocarps. In the latter instance, there is a linear sequence in the stages of development from aggregation to culmination.

If myxamoebae four to five days old are still capable of aggregating, it is rather surprising to find areas of a culture which are free of aggregates. When viewed under the microscope, the myxamoebae in these areas appear much as they do in other parts of the culture, with none of the features of dead or dying cells such as a high degree of vacuolation and Brownian movement. What prevents these cells from completing the life cycle? A partial answer to this question is given in the results of some preliminary experiments. For example, the excision and transfer of a center of aggregation to an area populated by these cells elicits, at best, a very weak chemotactic response. Assuming that the transplants are active producers

of an attractant (acrasin), this observation would indicate a defect in the chemotactic response mechanism. On the other hand, if such cells are collected, resuspended and deposited as isolated populations on a fresh non-nutrient substrate, they respond by forming pseudoplasmodia and sorocarps of perfectly normal appearance. This observation, taken together with the fact that charcoal is effective in stimulating center formation, suggests that there is something present in the environment and removable by charcoal which is responsible for inhibiting aggregation.

Effect of certain agents on aggregation density

It is evident from the material presented above that in *P. pallidum,* light does have a very real and dramatic effect upon the initiation of cell aggregation and upon the density of such aggregations. What is much less evident is how light acts to achieve this effect. A hypothesis stating simply that light is required for aggregation

TABLE I

The effect of various agents on aggregation density. The first value in each column represents the mean number of centers per drop; the number of drops averaged is in parenthesis

Agent	Control	Experimental
1. Myxamoebae grown in the *dark*, harvested, washed and		
a) Incubated in the dark:		
Heat*	1.7 (40)	1.3 (40)
Light Cells+	1.5 (60)	1.5 (37)
CO_2‡	2.0 (20)	1.5 (20)
KOH**	1.0 (40)	1.1 (40)
Charcoal++	1.5 (40)	5.8 (40)
Mineral Oil‡‡	1.3 (40)	18.3 (40)
b) Incubated in the light:		
Heat	—	—
Light Cells	21.0 (19)	21.7 (20)
CO_2	23.5 (20)	13.5 (20)
KOH	23.5 (20)	23.0 (20)
Charcoal	16.7 (56)	26.2 (60)
Mineral Oil	15.8 (56)	43.2 (49)
2. Myxamoebae grown in the *light*, harvested, washed and		
a) Incubated in the dark—1.1 (37)		
b) Incubated in the light—23.4 (38)		

* Dark-grown myxamoebae were exposed for 15 and 30 minutes to a temperature of 33° C.

+ Dark-grown myxamoebae were combined in mixed populations with cells preincubated in the light for one or two hours, *i.e.*, sufficient to elicit aggregation. The proportions were approximately 20% "light"-grown to 80% "dark"-grown cells.

‡CO_2 was provided by placing pieces of dry ice, about one cubic centimeter in volume, in the lids of inverted cultures.

** Large well slides containing several milliliters of KOH solution (5% and 10%) were placed in the lids of inverted cultures.

++ The charcoal was placed in aluminum cups which were either embedded into the agar to form a receptable or placed in the lids of inverted cultures.

‡‡ After the excess liquid from the drop was absorbed into the agar, the myxamoebae were covered with a layer of mineral oil.

is untenable since aggregation can and does occur in its absence. There are a number of possible hypotheses which could account for the effect of light on aggregation. Among these are (1) the destruction, or inactivation, of acrasin, and (2) the alteration in the response of myxamoebae to chemotactic stimuli. The notion most readily accessible to experimentation, and that suggested, in part, by the preceding experiments, is that light operates by changing in some way the relationship between the process of aggregation and an aggregation (or center) suppressing factor produced by the cells and present in the environment. For example, light might limit the production of or, perhaps, destroy such a factor, thereby permitting aggregation to occur. This view is consistent with the recent proposals of Bonner and Dodd (1962) and Bonner and Hoffman (1963). To test this notion, and some others, certain experiments were performed, the results of which are presented in Table I. Because there is some variation in the behavior of myxamoebae from one experiment to another, values are compared only within the same experiment or group of experiments. In evaluating these results, it is assumed that if a suppressor or "spacing substance" is involved, then its removal should lead to an increase in the number of centers.

To summarize these results on a point-by-point basis:

Heat appears not to be an effective replacement for light. This observation is of some importance since it tends to eliminate from these experiments one of the factors, a rise in temperature, reported by Raper (1940) as important in increasing the density of aggregation in *Dictyostelium discoideum*.

Myxamoebae illuminated sufficiently to initiate aggregation do not impart this ability to dark-incubated cells and appear themselves to be limited by the associated "dark cells" from forming centers. This observation, in and of itself, is a rather strong argument against a hypothesis based simply on the light-induction of centers.

While CO_2 had little effect on dark-incubated cultures, it sharply reduced the number of aggregates formed in the light. This is in agreement with the observations of Bonner and Hoffman (1963) on *D. mucoroides*.

KOH at concentrations up to 20% had little or no effect on either dark- or light-incubated cultures. The reciprocal might have been expected based upon the above observation.

As in Bonner and Hoffman's experience, charcoal does increase the density of aggregations among myxamoebae incubated in the light. More interestingly, the presence of charcoal in dark-incubated cultures increased the number of centers to about four times the control value. This result would be consistent with the presence of a center-inhibiting factor, or suppressor, which exists, at least in part, in the gaseous phase.

Mineral oil provides the sharpest change in the behavior of the myxamoebae. In illuminated cultures, the density of aggregation was increased about three-fold (*cf.* Bonner and Hoffman, 1963), while in the dark there was a *14*-fold increase. That is to say, in the dark and under mineral oil a density of aggregation was reached which is normally achieved only in the light! How this effect is brought about is still unclear, but it could possibly result from the adsorption by mineral oil of a suppressive factor.

Growing myxamoebae in the light as opposed to darkness does not alter their behavior in terms of aggregative density.

Discussion

A number of hypotheses could be invoked to account for the effect of light on the initiation of cell aggregation and on aggregation density in *Polysphondylium pallidum*. Of these, only one was subjected to any sort of experimental evaluation. The major feature of this particular hypothesis is that light acts, in some way, to prevent a center-suppressing factor present in the environment from inhibiting aggregation. This might occur by light limiting the production of the inhibiting factor or perhaps by destroying it. Another possibility would be for light to render myxamoebae less sensitive to suppression or more sensitive to a lower level of stimulation. In any case, the net effect would be an increase in the number of centers among myxamoebae exposed to light. What, then, are the data supporting this hypothesis?

As evidence for the presence of a suppressor (in this case one existing in the gaseous phase), one may recall that an agent such as charcoal in both isolated and *in situ* populations increases, as does light, the density of aggregation centers. Furthermore, in cultures grown *in situ*, sorocarp formation and aggregation induced by charcoal follow much the same pattern as that of light induction, *i.e.*, in both a zone of morphogenetic activity is formed near the periphery of the expanding colony. Another piece of evidence, albeit indirect, is the appearance of sorocarps at or near the point of inoculation in cultures grown in the dark. Bonner and Hoffman (1963) discuss in some detail situations in which a relatively great air space above the myxamoebae permitted center formation where none might have occurred under more confined conditions, hence implying the accumulation of a center-limiting factor in the atmosphere. Cell aggregation which occurs at the point of inoculation before the culture has attained substantial dimensions is possibly a good example of this. Finally, cultures which are exposed to charcoal aggregate more quickly than do unexposed cultures—a response also seen when one compares light-treated cultures with those maintained in darkness.

A diminution in the effectiveness of light with time has been indicated by the experiments illustrated in Figures 1 and 2, and, indirectly, by the experiments presented in Figure 3. On the basis of the hypothesis presented above, this diminution could be accounted for by the accumulation of the suppressing factor(s). That is, the longer the culture remains in the dark, the more suppressor accumulates and the more difficult becomes the process of cell stimulation. On the other hand, if cultures are illuminated shortly after they cease feeding, *i.e.*, as soon as they become "ready" to aggregate, little suppressor would have accumulated, and, as a consequence, a small amount of stimulant, *e.g.* less light, could give rise to a comparatively great response. In fact, this is quite nearly the type of behavior represented in Figures 1 and 2 where the greatest sensitivity to light is evident early within the incubation period.

Is there evidence, beyond that already presented, for a suppressor type of hypothesis? Bonner and Hoffman (1963) found that the major species of the Dictyosteliaceae could be separated upon their aggregative behavior into two groups: gas-sensitive and gas-insensitive. In their experiments the gas-sensitive species, *Polysphondylium pallidum*, *P. violaceum*, and *Dictyostelium mucoroides*, showed vastly increased aggregation densities in the presence of charcoal and under mineral

oil. *Dictyostelium purpureum* and *D. discoideum*, the gas-insensitive species, are invariant to these factors. Therefore, for at least three of the five major species there are indications of a center-limiting factor in the gaseous phase of the environment.

Shoopman (1963) found in *D. deminutivum* (*sp. nov.*, to be described) that cell aggregation and culmination were often retarded in standard Petri dish cultures, but proceeded normally when charcoal was added or when the glass lid was replaced by a porous clay top. Shaffer (1961) noted that light applied to dark-incubated cultures of *P. violaceum* brought about a burst of aggregative activity. This response normally occurred within 20 minutes after exposure, which correlates well with the quick response seen in *P. pallidum*. More germane are his observations on the aggregative behavior of myxamoebae sandwiched between two thin layers of agar. In the dark, cultures of this type usually either failed to aggregate or aggregation was delayed for periods of several days, *except* in those cultures which had trapped air bubbles, or in which the myxamoebae came to lie in close proximity to the edge of the overlying piece of agar. Here, again, the implication is for the presence of a center-suppressing factor which exists in the gas phase.

It would be well to point out another observation made by Shaffer (1961) and confirmed during the course of this investigation. Under agar, where centers are normally inhibited or their numbers reduced, myxamoebae are still capable of forming quite extensive streams. Thus, while center formation is impaired, the ability to undergo the physiological changes requisite to stream formation has not been impaired.

The factor postulated as being involved in center formation has been termed a "suppressor," which may be similar if not identical to Bonner and Hoffman's "spacing substance." The latter term was used to connote a gaseous substance which determines in some rather precise way the spacing of aggregation centers, *i.e.*, the distance between one center and another. During the present investigation, evidence has been obtained which points to the presence in *P. pallidum* of a gaseous center-suppressing factor that is produced by the myxamoebae and may determine whether a center *will* or *will not* develop. Nothing with certainty can be said as to whether or not this factor determines the distance between two centers. Because of this uncertainty, the rather specific term "spacing substance" has been avoided and the more general expression "suppressor" employed in presenting the results of this study.

As to the nature of the gas, Bonner and Hoffman (1963) have presented evidence that it may be CO_2. In this work, it has been found, in agreement with the above, that CO_2 will reduce the number of centers formed by a population of myxamoebae as, in contrast, charcoal will enhance the number. The only incongruity is that KOH, normally a good CO_2 absorbent, was only marginally effective in increasing the number of centers and then only in the dark (Table I). Consequently, at least for *P. pallidum* strain WS-320, it would seem unlikely that CO_2 is the naturally occurring center-suppressing factor.

Only a single explanation, the removal or inactivation of a suppressor, has been offered in this discussion to account for the effect of light on cell aggregation in *P. pallidum*. One need only to recall that two major species, *D. discoideum* and *D. purpureum*, are both light-sensitive *and* gas-insensitive to realize how inadequate

is this simple explanation. We certainly cannot say at this time that the mechanism by which light effects aggregation in these two species may not be operative in *Polysphondylium pallidum* as well.

I wish to thank Professor Kenneth B. Raper for providing the laboratory facilities required for this investigation, and for his help and encouragement, particularly in the preparation of this manuscript.

This study was supported in part by a Training Grant from the National Institutes of Health (5-T1-GM-686).

Summary

Experiments have been performed on the effect of light on cell aggregation and aggregation density in *Polysphondylium pallidum*, strain WS-320. The results of these experiments may be summarized as follows:

1. Cell aggregation occurs more quickly and with a greater density of aggregation centers in cultures incubated in the light than in comparable cultures incubated in the dark.

2. Myxamoebae grown in the dark in liquid culture and deposited on agar are most sensitive to light within the first few hours of incubation.

3. Beyond the initial period of sensitivity, there is a diminution in response resulting in an almost complete absence of light-induced aggregation.

4. Little variation in response occurred when myxamoebae were exposed to light for periods of from one to 15 minutes, indicating a fairly sensitive light response mechanism.

5. In cultures grown *in situ*, exposure to the light following dark incubation induces aggregation in two- to three-day-old myxamoebae. Older myxamoebae fail to display any sign of morphogenetic activity in response to light; they do respond, however, in the presence of charcoal.

6. Of certain physical and chemical agents tested for their effect on aggregation, charcoal and mineral oil were found to increase aggregation density in both light and dark, while CO_2, in the light, had the opposite effect.

The similarity in response achieved by charcoal and mineral oil, and by light, suggests that the action of light may be to change the relationship between aggregation and a center-suppressing factor that accumulates in the environment. In the light, aggregation occurs possibly because light limits the production of or destroys the center-suppressing factor, or renders the cells less sensitive to it. In the absence of light, aggregation may be inhibited by this substance while the cells are shunted in the direction of microcyst formation. If, in the latter situation, charcoal or mineral oil were added prior to actual cyst formation, the suppressor would be absorbed and aggregation could occur.

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